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# Sex-specific regulation of growth plate chondrocytes by estrogen is via multiple MAP kinase signaling pathways

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**Abstract**

Both male and female rat growth plate cartilage cells possess estrogen receptors (ERs), but 17β-estradiol ($E_2$) activates protein kinase C (PKC) and PKC-dependent biological responses to $E_2$ only in cells from female animals. PKC signaling can elicit genomic responses via mitogen activated protein kinase (MAPK) and $E_2$ has been shown to activate ERK MAPK in many cells, suggesting that MAPK may play a role in growth plate chondrocytes as well. We tested if $E_2$ increases MAPK activity and if so, whether the response is limited to female cells, if it is PKC-dependent, and if the mechanism involves traditional ER pathways. We also determined the contribution of MAPK to the biological response of growth plate chondrocytes and assessed the relative contributions of ERK, p38 and JNK MAPKs. Female rat costochondral cartilage cells were treated with $E_2$ and MAPK-specific activity determined in cell layer lysates. The mechanism of MAPK activation was determined by treating the cells with $E_2$ conjugated to bovine serum albumin ($E_2$-BSA) to assess if membrane receptors were involved; stereospecificity was determined using 17α-estradiol; PKC and phospholipase C (PLC) dependence was determined using specific inhibitors; and the ER agonist diethylstilbestrol, the ER antagonist ICI 182780, and tamoxifen were used to assess the role of traditional ER pathways. $E_2$ regulation of ERK1/2 MAPK was assessed and the relative roles of ERK1/2, p38 and JNK MAPKs determined using specific inhibitors. $E_2$ caused a rapid dose-dependent activation of MAPK that was greatest in cells treated for 9 min with $10^{-9}$ M hormone; activity remained elevated for 3 h. $E_2$'s effect on MAPK was stereospecific and comparable to that of $E_2$-BSA. It was insensitive to DES and ICI 182780, dependent on PKC and PLC, blocked by tamoxifen and it did not require gene transcription or translation. $E_2$ had no effect on ERK1 or ERK2 mRNA or protein but it caused a rapid phosphorylation of ERK1/2 at 9 min. Inhibition of ERK1/2 and p38 MAPK reduced the stimulatory effects of $E_2$ on alkaline phosphatase activity and [$^{35}$S]-sulfate incorporation. These results suggest that $E_2$ regulates MAPK through a sex-specific membrane-mediated mechanism that does not involve cytosolic ERs in a traditional sense and that ERK1/2 and p38 mediate the downstream biological effects of the hormone.


*Keywords:* 17β-Estradiol; Sex-specificity; MAP kinase; Growth plate chondrocytes; ERK; p38; JNK

## 1. Introduction

Estrogen ($E_2$) regulates cells through classical steroid hormone receptor mechanisms involving two receptors, ERα and ERβ. Both receptors have been found in plasma membranes of estrogen-sensitive cells [31,45], as have truncated forms [19,32], suggesting that they might be involved in the membrane-associated effects of the hormone. It is not clear how membrane-associated ERs participate in the mechanism of $E_2$ action, however. Recent studies examining $E_2$-dependent phosphorylation of the ERK family of mitogen-activated protein kinases (MAPKs), indicate that at least one mechanism involves the formation of membrane-associated heterodimers of ERα and ERβ [54].

Traditional cytosolic ERs are implicated in non-traditional steroid hormone mechanisms [34], but non-ER pathways may

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also play a role. $E_2$ causes rapid changes in intracellular $Ca^{++}$ [16,51,67], and in some instances this is unaffected by the ER antagonist ICI 182780 [62,77]. Even when ERα and ERβ are present, not all cells are responsive to the hormone. Although both male and female cells possess nuclear receptors for ERα and ERβ [2,44], some of the rapid responses to $E_2$, including activation of protein kinase C (PKC), are limited to cells from female animals [2,16,44]. Moreover, PKC activation in female cells is unaffected by the ER agonist diethylstilbestrol (DES) or the ER antagonist ICI 182780, but it is blocked by tamoxifen, which is a PKC inhibitor [46,60]. Estrogen that has been conjugated with bovine serum albumin ($E_2$-BSA) to prevent it from crossing the plasma membrane [80], also activates PKC and the biological responses that are dependent on PKC activation, but does not affect these biological responses in male cells [68,73]. The rapid effects of estrogen and $E_2$-BSA are also seen in breast cancer cell lines that lack ERα and ERβ [8]. These observations suggest that $E_2$ activates PKC signaling by a mechanism that is independent of these receptors in a traditional sense and there is now evidence that novel G-protein coupled receptors may be involved [26,53].

In female rat growth plate chondrocytes, $E_2$ activates PKC by rapidly increasing intracellular $Ca^{++}$ concentration through a capacitative entry mechanism [18], resulting in increased phosphatidylinositol-dependent phospholipase C (PLC) activity [58,70]. PKC activation can initiate a signaling cascade that results in changes in gene expression through MAPK [11,38]. The fact that ERK1/2 MAPK family has been shown to be activated by $E_2$ in osteoblasts [64], embryonic fibroblasts, and HeLa cells [28,29], suggests that it may also mediate effects of the hormone on growth plate chondrocytes. It is not known, however, if $E_2$ regulates MAPK in a sex-specific manner, whether MAPK is upstream or downstream of PKC activation, or if the effects of $E_2$ on MAPK are limited to ERK. $E_2$ has also been shown to activate p38 MAPK [62] and JNK MAPK [29], which like ERK are expressed in chondrocytes [1,24,33,37,39,49,61,76], raising the possibility that more than one form of the enzyme plays a role. The purpose of this study was to determine if the biological responses of growth plate chondrocytes to $E_2$ involve one or more of these signaling pathways, and if so whether they contribute to the sex-specific responses of these cells to this steroid hormone.

## 2. Materials and methods

### 2.1. Reagents

The following reagents were purchased from Sigma Chemical Company (St. Louis, MO): 17β-estradiol ($E_2$), 17β-estradiol-bovine serum albumin ($E_2$-BSA), 17α-estradiol, PD98059 (MEK inhibitor) [78], SB203580 (p38 inhibitor) [75], SP600125 (JNK inhibitor) [10], chelerythrine and H-7 (PKC inhibitors) [23,25], U73122 (phosphatidylinositol-specific PLC inhibitor) [4], actinomycin D (transcription inhibitor), cycloheximide (translation inhibitor), and indomethacin (cyclooxygenase inhibitor). The Biotrack p42/p44 MAPK assay kit was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM) was obtained from Cellgro, Inc. (Atlanta, GA), and fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). The protein content of each sample was determined using the bicinchoninic acid protein assay reagent [63] obtained from Pierce Chemical Co. (Rockford, IL). [$^{32}$P]-ATP, [$^{3}$H]-thymidine and [$^{35}$S]-sulfate were obtained from Perkin-Elmer (Boston, MA).

### 2.2. Chondrocyte cultures

The culture system used in this study has been described in detail previously [6]. Chondrocytes were isolated from the resting zone (reserve zone) and growth zone (prehypertrophic/upper hypertrophic cell zones) of the costochondral cartilage of 125 g female and male Sprague–Dawley rats (approximately 1 month old). For each batch of cells, the costochondral cartilages were dissected from 18 rats. The chondrocytes from these cartilages were combined and cultured in DMEM containing 10% FBS and 50 μg/ml vitamin C in an atmosphere of 5% $CO_2$ and 100% humidity at 37 °C. Sharp dissection was used to separate the cartilage layers. This is done under a dissecting scope, and the zones can be visualized as differences in opacity. Resting zone chondrocytes are primarily from the reserve zone, with some contamination from the uppermost region of the proliferative cell zone. The central portion of the proliferative cell zone is discarded to limit contamination. Growth zone cells are from the prehypertrophic and upper hypertrophic cell zones. The lower hypertrophic cell zone is discarded to limit contamination from osteoblasts. Some cells from the lowermost region of the proliferative cell zone may be included in the growth zone cell population. Fourth passage cells were used for all experiments, as prior studies have shown retention of differential phenotype, including extracellular matrix composition, plasma membrane and matrix vesicle fluidity and phospholipid composition, responses to cytokines, growth factors, hormones, the vitamin D metabolites 1α,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$, at this number of passages (see [5,7] for examples). Moreover, the behavior of the fourth passage cells in culture reflects the behavior of their zone of origin in vivo (see [14,15,56] for examples). Fourth passage RC and GC cells express mRNAs for Sox-9, aggrecan, cartilage oligomeric matrix protein (COMP), and type II collagen. They also express type I collagen as an adaptation to monolayer culture but they do not express osteocalcin. Typical of growth plate cartilage cells, they express mRNA for alkaline phosphatase and activity of this enzyme is greater in the GC cells, commensurate with their in vivo phenotype. While GC cells do possess mRNA for type X collagen, SDS-polyacrylamide gel electrophoresis of the extracellular matrix indicates that they do not produce the protein.

Each experiment was conducted so that there were six separate cultures for each variable tested, all cultures coming from one batch of cells. Each experiment was repeated multiple times, using cells from a different batch each time. Because our cells come from animals and not from an established cell line, there are differences in baseline values due to differences in media, the team doing the dissection, etc. To ensure that data from any one experiment were comparable to results from other experiments, we compared the results using treatment/control ratios. Using this approach, we had sufficient power in each experiment for statistical analysis (described below).

At confluence, media were removed and replaced with experimental medium containing estrogen at the concentrations indicated below. Although $E_2$-BSA is known to contain some free $E_2$, we did not remove free $E_2$ from the $E_2$-BSA used in the present study prior to its addition to the cultures. We previously showed that responses of the growth plate chondrocytes to unfiltered $E_2$-BSA were comparable to those seen in chondrocytes treated with filtered $E_2$-BSA [73].

### 2.3. Regulation of MAPK by $E_2$ and $E_2$-BSA

#### 2.3.1. MAPK-specific activity

MAPK-specific activity was determined using the Biotrack p42/p44 MAPK assay kit following the manufacturer's directions. Confluent cultures of male and female resting zone and growth zone chondrocytes were treated with $10^{-10}$ to $10^{-7}$ M $E_2$ for 9 or 90 min. To determine time course, cultures were treated with $10^{-9}$ M $E_2$ for 9, 90, 180 or 270 min. Stereospecificity was determined by treating female cells with $E_2$ or its stereoisomer 17α-estradiol ($10^{-10}$ to $10^{-7}$ M) for 9 min. Female growth zone and resting zone cells were also treated with $E_2$-BSA ($10^{-10}$ to $10^{-7}$ M) to determine if the increase in MAPK was membrane mediated. Control cultures were treated with media containing the ethanol vehicle. After the appropriate incubation period, cell layers were washed with PBS and lysed in solubilization buffer [50 mm Tris–HCl (pH 7.5), 150 mm NaCl, 5 mm EDTA, and 1% Nonidet P-40] for 30 min

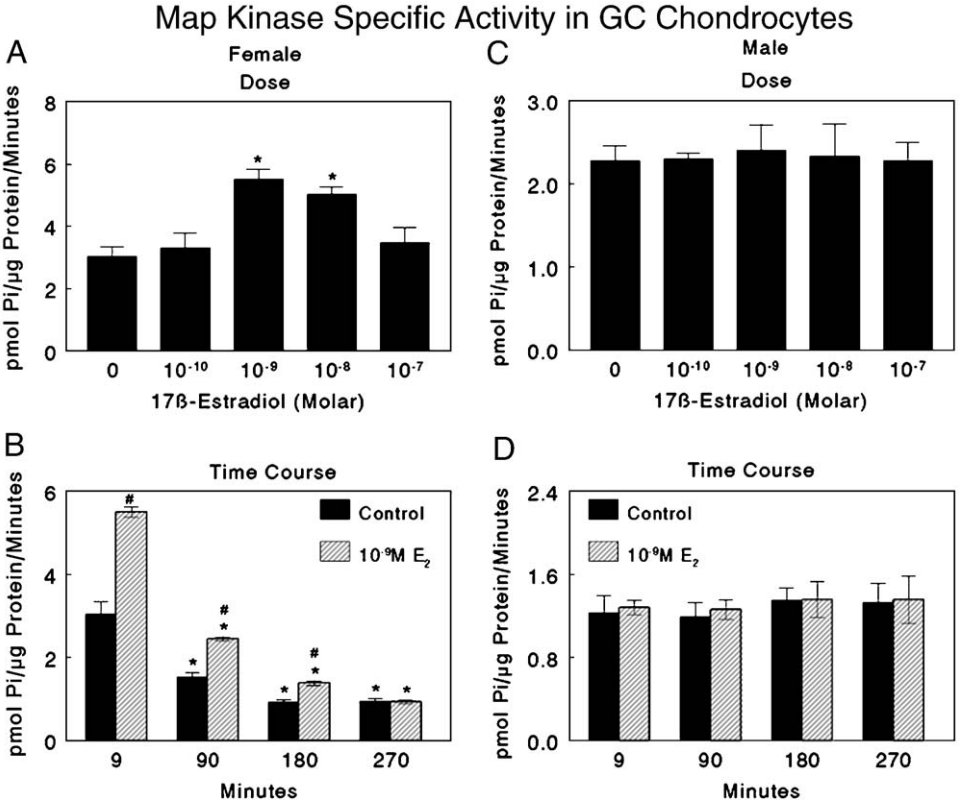


Fig. 1. Effect of 17β-estradiol on MAPK-specific activity of confluent growth zone chondrocyte cultures isolated from female and male rats. Cultures were treated with $10^{-10}$ to $10^{-7}$ M 17β-estradiol for 9 min (A, female cells; C, male cells), or for 9–270 min with $10^{-9}$ M 17β-estradiol (B and D). MAPK-specific activity was measured in cell layer lysates as described in Materials and methods. Values are from a representative experiment and are the mean ± S.E.M. for six independent cultures. The experiment was repeated two additional times with nearly identical results. $^{*}P<0.05$ vs. control (A and C) or 9 min (B and D); $^{\#}P<0.05$ vs. 17β-estradiol treatment (B and D).

on ice. The cell layer lysates were assayed for protein content [55] and MAPK activity.

*2.3.2. ERK1/2 phosphorylation*

To determine if 17β-estradiol regulated the levels of ERK1/2 protein or activated MAPK activated by tyrosine phosphorylation [11], cell culture lysates were examined by Western blot using specific antibodies to non-phosphorylated and phosphorylated p42/p44 MAPK. Cell culture lysates were prepared from confluent, fourth passage growth zone cell cultures that had been treated for 9 or 90 min with $10^{-9}$ to $10^{-7}$ M $E_2$ and were resolved on 10% SDS-polyacrylamide gels. Blots of the gels were probed with 1:5000 dilutions of rabbit polyclonal antibodies to phosphorylated p42/p44 or non-phosphorylated p42/p44 (Promega Corp., Madison, WI), followed by 1:5000 dilutions of alkaline phosphatase-conjugated anti-rabbit IgG1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactive bands were visualized using the nitro blue tetra-zolium/5-bromo-4-chloro-3-indolyl-phosphate reagent (Sigma).

*2.3.3. Northern blot analysis*

To quantify the effects of $E_2$ on mRNA levels for ERK1 and ERK2, we performed Northern blot analysis. Chondrocytes were treated with $10^{-10}$ to $10^{-7}$ M $E_2$ for 9, 90 and 270 min. Total RNA for untreated and $E_2$-treated female growth zone and resting zone chondrocytes was isolated with TRIzol, quantified spectrophotometrically, separated on a 1% denaturing agarose gel, and transferred to a positively charged nylon membrane (Ambion, Inc.) with the Turboblotter System (Schleicher and Schuell, Inc., Keene, NH). Northern blots were hybridized with ERK1, ERK2, and glyceraldehyde-3-phosphate dehydrogenase-strippable $^{32}$P-labeled anticoding RNA probes using the NorthernMax Kit (Ambion, Inc.). ERK1 and ERK2 DNA templates were synthesized from sequenced RT-PCR products amplified with modified antisense primers with the T7 promoter sequence, 5′-TAA TAG GAC TCA CTA TAG GGA GG-3′, attached to the 5′-end of the antisense primers. Anticoding RNA probes were synthesized with the Strip-EZ T7 Kit (Ambion, Inc.). Northern blots were analyzed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

*2.3.4. Requirement for gene expression and protein synthesis*

To determine whether new gene expression or protein synthesis is required for $E_2$-dependent stimulation of MAPK activity, female growth zone and resting zone chondrocytes were treated with 1, 10, or 100 μM actinomycin D to block transcription or with 1, 10, or 100 μM cycloheximide to block translation [48]. These doses were selected based on previous studies using the costochondral growth plate chondrocyte model [72].

*2.3.5. Requirement for nuclear estrogen receptors*

To determine if traditional nuclear estrogen receptor mechanisms are required for activation of MAPK, female growth zone and resting zone chondrocytes were incubated with an estrogen receptor antagonist, ICI 182780 or with an estrogen receptor agonist, diethylstilbestrol (DES) [74]. Confluent cultures were treated with vehicle or $10^{-9}$ M $E_2$ in the presence and absence of $10^{-9}$ to $10^{-7}$ M antagonist or agonist. MAPK-specific activity was measured at 9 min.

*2.3.6. Protein kinase C*

17β-Estradiol mediates its effects on growth zone cells via PKC signaling pathways [70]. To determine whether $E_2$-dependent activation of MAPK involves PKC, PKC activity was blocked using chelerythrine (0.1, 1, or 10 μM) or with $10^{-9}$ to $10^{-7}$ M tamoxifen, as described by us previously [8,60]. Female growth zone and resting zone chondrocyte cultures were incubated for 9 and 90 min in control medium or medium containing $10^{-9}$ M $E_2$ in the presence and absence of the inhibitor.

*2.3.7. Phospholipase C*

PLC mediates the effects of $E_2$ on PKC in growth zone cell cultures [70]. Its involvement in the effect of $E_2$ on MAPK was assessed by using U73122, an

inhibitor of phosphatidylinositol specific (PI)-PLC [4], at 0.1, 1.0, or 10 μM. Female growth zone and resting chondrocytes were incubated for 9 and 90 min in control medium or medium containing $10^{-9}$ M $E_2$, with or without U73122 and MAPK activity was determined.

### 2.4. Role of MAPK in mediating the biological response to 17β-estradiol

Female growth zone and resting zone chondrocytes were used for these experiments. To block the effects of ERK1/2 MAPK, cells were treated with the MEK inhibitor PD98059 [78]. Two sets of experiments were performed. Cultures were treated with vehicle (control) or $10^{-9}$ M $E_2$ in the presence of 1, 10, or 100 μM PD98059. In the second set of experiments, cultures were treated with 0 or 100 μM PD98059 in the presence of vehicle or $10^{-10}$ to $10^{-7}$ M $E_2$. To block the effects of p38 MAPK, cells were treated with vehicle or $10^{-9}$ M $E_2$ in the presence of $10^{-7}$ to $10^{-5}$ M SB203580 [75]. To block the effects of JNK MAPK, cells were treated with vehicle or $10^{-9}$ M $E_2$ in the presence of $10^{-7}$ to $10^{-5}$ M SP600125 [10]. In each case, cultures were incubated with $E_2 \pm$ inhibitor for 24 h.

#### 2.4.1. [$^3$H]-thymidine incorporation

17β-Estradiol inhibits [$^3$H]-thymidine incorporation by resting zone and growth zone chondrocytes [40]. To determine whether this response is mediated by MAPK, DNA synthesis was estimated by measuring [$^3$H]-thymidine incorporation into trichloroacetic acid-insoluble cell precipitates as described previously [40]. Quiescence was induced by incubating preconfluent cultures for 48 h in DMEM containing 1% FBS. The medium was then replaced with DMEM containing 1% FBS alone (control), $E_2$, and inhibitor (or appropriate vehicles). Two hours before harvest, [$^3$H]-thymidine was added.

#### 2.4.2. Alkaline phosphatase-specific activity

17β−Estradiol stimulates alkaline phosphatase-specific activity in growth zone and resting zone chondrocyte cultures [40]. To determine whether this effect was mediated by MAPK, confluent cultures were treated with 17β-estradiol and inhibitor for 24 h. Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline (EC 3.1.3.1)]-specific activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2, as described previously [9,22,57].

#### 2.4.3. Proteoglycan sulfation

17β-Estradiol causes an increase in [$^{35}$S]-sulfate incorporation in growth zone chondrocyte cultures [40]. To determine whether this is mediated by MAPK, proteoglycan synthesis was assessed by measuring [$^{35}$S]-sulfate incorporation by confluent cultures as described by us previously [42,47]. At confluence, cells were treated with fresh medium containing $E_2$ plus inhibitor for 24 h. Four hours before harvest, 50 μl DMEM containing 18 μCi/ml [$^{35}$S]-sulfate and 0.814 mM carrier sulfate were added to each culture. At harvest, the conditioned media were removed, the cell layers (cells and matrix) were collected, and the amount of [$^{35}$S]-sulfate incorporated was determined as a function of cell layer protein.

### 2.5. Statistical management of data

For each experiment, each value represents the mean ± S.E.M. of the cell layers of six independent cultures. Statistical significance was determined by ANOVA. Bonferroni's modification of the t test was used for post hoc testing. $P<0.05$ was considered significant. Each experiment was repeated two or more times to ensure the validity of the data. The data presented are from a single representative experiment. Effects of $E_2$ on MAPK were similar in growth zone and resting zone chondrocyte cultures. For this reason, only the results for growth zone cells are presented as figures for many of the experiments below.

## 3. Results

MAPK activity was sensitive to 17β-estradiol, but only in female cells. In female growth zone cells, $E_2$ caused a biphasic increase in MAPK-specific activity that was greatest at $10^{-9}$ M (Fig. 1A). The effect was maximal at 9 min, although activity remained elevated over controls through 180 min (Fig. 1B). In contrast to the stimulatory effects of $E_2$ on female cells, male cells exhibited no response either as a function of $E_2$ concentration (Fig. 1C), or time (Fig. 1D). Resting zone cells responded to $E_2$ in a similar manner, with peak activity in female cells at $10^{-9}$ M hormone and at 9 min and no effect on MAPK in male cells (data not shown).

The effect of $E_2$ on MAPK was nongenomic. Growth zone and resting zone chondrocytes both expressed mRNA for ERK1/2 (p42/p44) based on RT-PCR (data not shown) and Northern blot analysis (Fig. 2A), and the levels of expression were similar in both types of cells. Treatment with $E_2$ had no effect on mRNA levels for either form of the enzyme in either type of cell. Inhibition of gene transcription with actinomycin D or of protein synthesis with cycloheximide had no effect on the increase caused by $E_2$ (data not shown). Western blots of total ERK1/2 demonstrated that the total amount of this MAPK was unchanged after treatment of female growth zone cells for either 9 or 90 min (Fig. 2B). However, phosphorylated ERK1/2 was increased and peak levels were seen in cells treated with $10^{-9}$ M for 9 min. The effect on resting zone cells was comparable (data not shown).

$E_2$ activated MAPK via a stereospecific mechanism. The inactive stereoisomer 17α-estradiol had no effect on MAPK activity in female growth zone cells (Fig. 3A) or female resting zone cells (Fig. 3B). The increase in MAPK was also

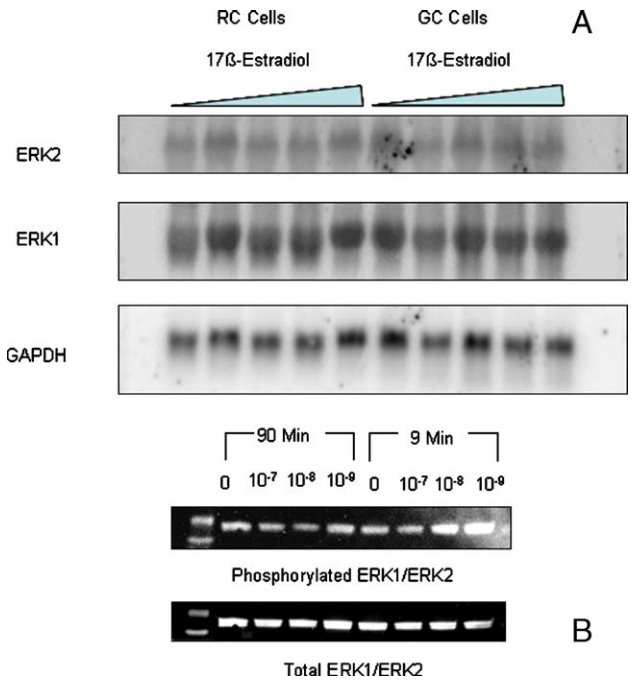


Fig. 2. Effect of $E_2$ on ERK1 and ERK2 in rat costochondral growth plate chondrocytes. (A) Confluent cultures of resting zone (RC) cells and growth zone (GC) cells were treated with vehicle or $10^{-10}$ to $10^{-7}$ M $E_2$ for 9 min. mRNA was isolated and levels of ERK1 and ERK2 mRNA determined via Northern blots using GAPDH as a control. (B) Growth zone chondrocytes were treated for 9 or 90 min with vehicle or $10^{-9}$ to $10^{-7}$ M $E_2$. Levels of phosphorylated ERK1/2 and total ERK were determined by Western blot analysis.

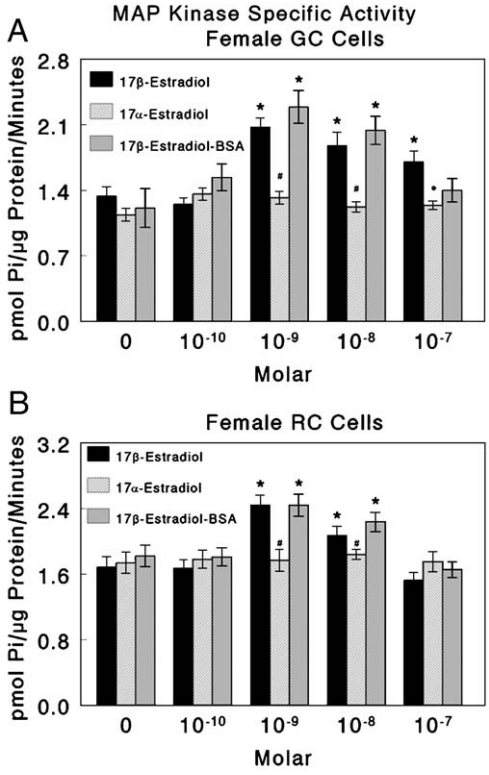


Fig. 3. Effect of 17α-estradiol, 17β-estradiol, and 17β-estradiol-BSA on MAPK-specific activity of confluent growth zone and resting zone chondrocyte cultures isolated from female rats. Confluent, female growth zone (GC) cells were treated with $10^{-10}$ M to $10^{-7}$ M estrogen for 90 min (A). Identical experiments were performed with female resting zone (RC) cells (B). MAPK-specific activity was measured in cell layer lysates as described in Materials and methods. Values are means±S.E.M. for six independent cultures. Data presented are from one experiment. The experiment was repeated two additional times with nearly identical results. *$P<0.05$ vs. control; $^{\#}P<0.05$ vs. 17β-estradiol treatment.

membrane-dependent. Treatment with $E_2$-BSA had the same stimulatory effect on enzyme activity in growth zone and resting zone cell cultures as was seen following treatment with the non-conjugated form of the hormone.

MAPK activation did not involve traditional estrogen receptor pathways. Treatment of growth zone cells (Table 1) or resting zone cells (data not shown) with the ER agonist DES or the ER antagonist ICI 182780 had no effect on $E_2$-dependent MAPK activity. However, both $E_2$ and $E_2$-BSA work via a tamoxifen-dependent mechanism. Tamoxifen caused a dose-dependent reduction in activation of MAPK by $E_2$ or $E_2$-BSA but sensitivity to the inhibitor differed (Fig. 4). The effect of tamoxifen on $E_2$-treated growth zone cells was less robust than on cultures treated with $E_2$-BSA. This was also true for resting zone cells (data not shown).

$E_2$ increased MAPK by a mechanism involving PKC and PLC. Treatment of female growth zone cells with the PKC-inhibitor chelerythrine caused a dose-dependent decrease in MAPK-specific activity (Fig. 5A). This effect was evident at all time points examined (Fig. 5B). Similarly, treatment of the cells with the PLC-inhibitor U73122 inhibited E2-dependent MAPK in a dose-dependent manner (Fig. 5C) at all time points

Table 1
Effect of ER agonist diethylstilbestrol (DES) and the ER antagonist ICI 182780 on MAP kinase-specific activity in cultures treated for 9 min with $10^{-9}$ M 17β-estradiol ($E_2$) or 17β-estradiol-BSA ($E_2$-BSA)

| Treatment | MAP activity (pmoles $PO_4$/μg protein/minute) | | |
|---|---|---|---|
| DES | Control | $E_2$ | $E_2$-BSA |
| 0 | 4.11±0.49 | 6.56±0.60* | 7.15±0.44* |
| $10^{-9}$ M | 4.20±0.48 | 6.37±0.63* | 6.63±0.65* |
| $10^{-8}$ M | 5.04±0.13 | 7.31±0.53* | 6.69±0.52* |
| $10^{-7}$ M | 4.83±0.17 | 6.30±0.29* | 6.88±0.31* |
| *ICI* 182780 | | | |
| 0 | 4.72±0.30 | 6.23±0.30* | 6.30±0.16* |
| $10^{-9}$ M | 4.84±0.25 | 6.24±0.30* | 6.13±0.31* |
| $10^{-8}$ M | 5.16±0.43 | 7.06±0.33* | 5.69±0.59* |
| $10^{-7}$ M | 4.62±0.41 | 6.11±0.23* | 6.17±0.19* |

Data are means±S.E.M. for 6 independent cultures and are from one of two experiments, both with comparable results. *$P<0.05$, with estrogen vs. without estrogen (Control).

(Fig. 5D). This was the case for resting zone cells as well (data not shown).

DNA synthesis in growth plate chondrocyte cultures was mediated by a mechanism involving ERK1/2. Treatment of the cells with PD98059 caused a dose-dependent decrease in [$^3$H]-thymidine incorporation in female growth zone cells (Fig. 6A) and resting zone cells (data not shown). The inhibitory effect of $E_2$ on DNA synthesis was affected by PD98059 only at the highest concentration of inhibitor. The inhibitory effect of PD98059 on DNA synthesis was additive with that of $E_2$ on DNA synthesis was additive with that of $E_2$ (Fig. 6B), suggesting that different mechanisms were involved.

ERK1/2 MAPK-mediated biological responses of female growth zone chondrocytes to $E_2$. PD98059 blocked the stimulatory effect of $E_2$ on alkaline phosphatase activity. The inhibitor had no effect on enzyme activity in control cultures of female growth zone cells, but reduced the $E_2$-dependent increase in a dose-dependent manner (Fig. 7A) and at all stimulatory doses of the hormone (Fig. 7B). PD98059 reduced the effect of $E_2$ on [$^{35}$S]-sulfate incorporation as well. The

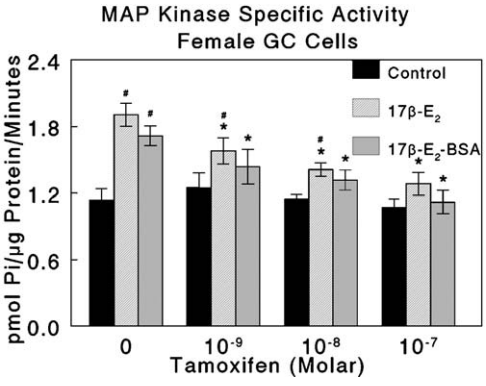


Fig. 4. Effect of tamoxifen on estrogen-dependent MAPK-specific activity in confluent growth zone chondrocyte cultures isolated from female rats. Cells were treated for 90 min with $10^{-9}$ M to $10^{-7}$ M tamoxifen in the presence of $10^{-9}$ M 17β-estradiol or 17β-estradiol-BSA. Values are means±S.E.M. for six independent cultures. Data are from one of two experiments, both with comparable results. *$P<0.05$ vs. control; $^{\#}P<0.05$ vs. tamoxifen treatment.

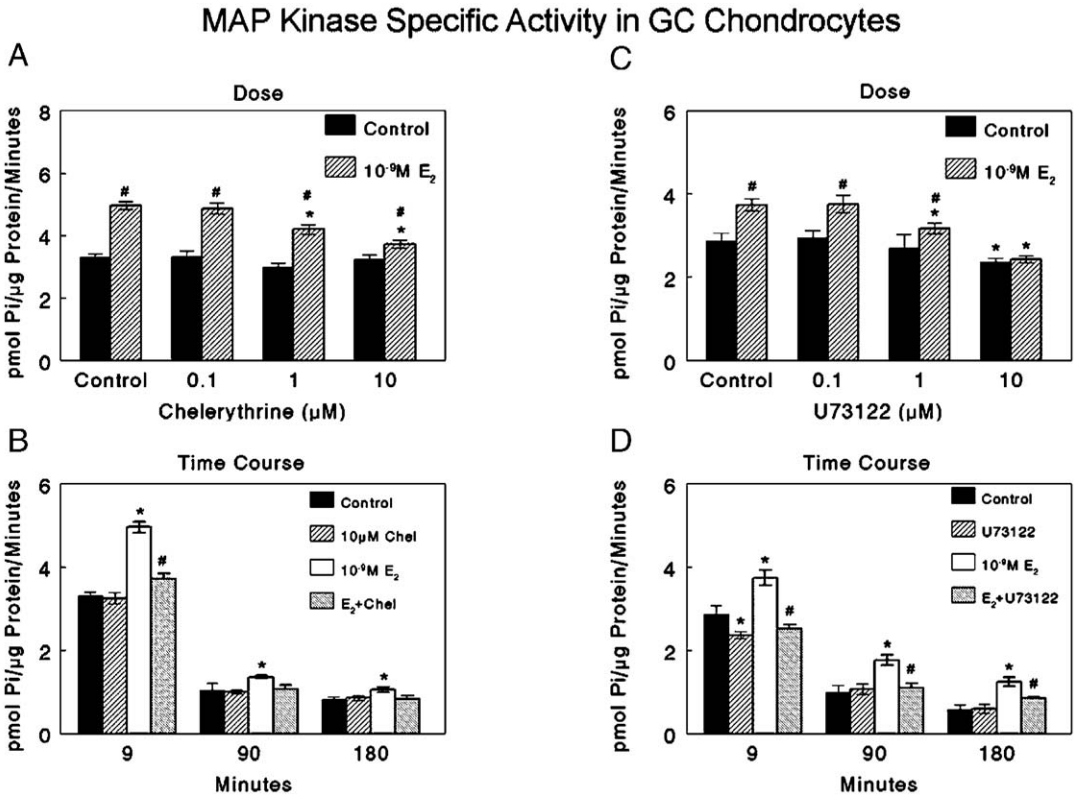


Fig. 5. Effect of chelerythrine and U73122 on estrogen-dependent MAPK-specific activity in cultures of confluent growth zone chondrocytes from female rats. Cells were treated for 90 min with 0.1, 1, or 10 μM chelerythrine (A) or U73122 (B) in the presence of $10^{-9}$ M 17β-estradiol. Time course experiments (B, chelerythrine; D, U73122) were also performed at time points of 9, 90, and 180 min. In these experiments, cultures were treated with either $10^{-9}$ M 17β-estradiol, inhibitor, or with $10^{-9}$ M 17β-estradiol and inhibitor to examine any combined effects. Values are the mean ± S.E.M. for six cultures from one experiment. *$P<0.05$ vs. control; #$P<0.05$ vs. inhibitor treatment.

effects were dose-dependent (Fig. 7C) and were seen at all stimulatory concentrations of the hormone (Fig. 7D), although this may have been due at least in part to a general regulation of proteoglycan synthesis via this signaling pathway.

Part of the $E_2$ effect on growth plate chondrocytes was via p38 MAPK. Inhibition of this family of MAPKs reduced [$^3$H]-thymidine incorporation in growth zone cells and at the highest concentration, was additive with $E_2$ (Fig. 8A), suggesting that separate mechanisms were involved. SB203580 also blocked the stimulatory effect of $E_2$ on alkaline phosphatase (Fig. 8B) and on [$^{35}$S]-sulfate incorporation (Fig. 8C). The inhibitor altered response of resting zone cells in a similar manner (data not shown).

DNA synthesis was mediated by JNK MAPK (Fig. 9A). SP600125 caused a dose-dependent decrease in [$^3$H]-thymidine incorporation in growth zone cells and at the highest concentration, blocked the response of the cells to $E_2$. SP600125 had no effect on alkaline phosphatase activity (Fig. 9B) or [$^{35}$S]-sulfate incorporation (Fig. 9C) in control cultures or in cultures treated with $E_2$. Similar responses were observed in resting zone cells (data not shown).

## 4. Discussion

This study shows that MAPK is regulated by $E_2$ in growth plate chondrocytes in a sex-specific manner that does not depend on the state of maturation of the cell in the endochondral pathway. Both resting zone cells and growth zone cells exhibited comparable responses to the hormone, as noted previously for PKC [70]. However, only female cells exhibited an increase in MAPK activity when treated with $E_2$. The increase depended on increases in PKC, based on inhibition by two PKC inhibitors, chelerythrine and tamoxifen. The increase also depended on PLC, based on inhibition by the phosphatidylinositol-specific inhibitor U73122. Both PKC and PLC have been shown to be stimulated by $E_2$ only in female cells [58,70]. In addition, these prior studies also showed that $E_2$ increases PKC via a PLC-dependent pathway [16], suggesting that $E_2$ acts on MAPK via a PLC-PKC signaling pathway.

As noted previously for PLC and PKC, the effects of $E_2$ on MAPK are membrane-dependent based on the observation that $E_2$-BSA elicits a comparable response. $E_2$-BSA has been shown to bind to surface receptors for 17β-estradiol [71], and remains extracellular [81]. Further support that specific receptors are involved is the observation that activation of MAPK was stereospecific; only treatment with 17β-estradiol and not 17α-estradiol resulted in increased enzyme activity. Our findings are commensurate with those in other laboratories demonstrating that $E_2$ acts via membrane receptors, including ERα and ERβ [19,31,32,45]. While the results indicate that $E_2$ acts through a membrane receptor-mediated mechanism, cytosolic estrogen

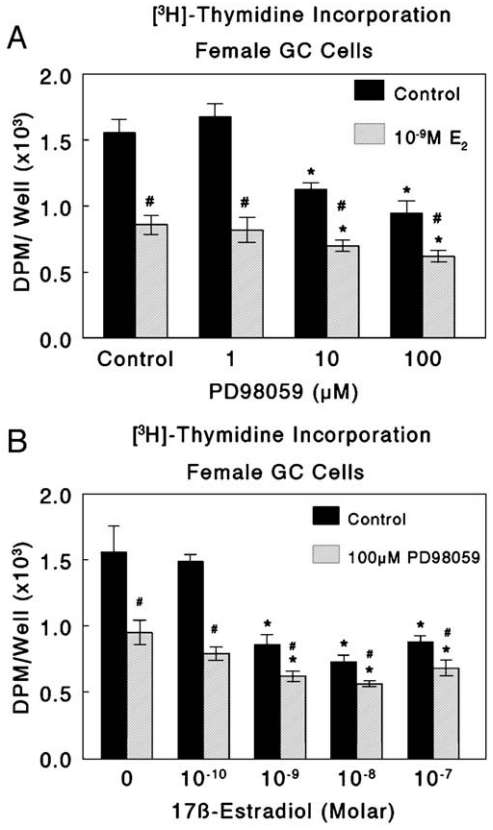


Fig. 6. Effect of PD98059 on [$^3$H]-thymidine incorporation in pre-confluent cultures of female rat growth zone (GC) chondrocytes. Cells were treated for 24 h with 1, 10 or 100 μM PD98059 in the presence of $10^{-9}$ M 17β-estradiol (A). Alternatively, cells were treated with $10^{-9}$ to $10^{-7}$ M 17β-estradiol in the presence of 100 μM PD98059 (B). Values are means±S.E.M. for six independent cultures. Data are from one experiment of two experiments, both with comparable results. *$P<0.05$ vs. control; $^{\#}P<0.05$ vs. treatment with $10^{-9}$ M 17β-estradiol or 100 μM PD98059.

receptors may have played a role as well. Cytosolic ERα and a 46 kDa variant of ERα have been shown to interact directly with PKCα in osteoblasts, resulting in phosphorylation of c-Src [12].

It is unlikely that traditional nuclear receptor pathways were involved. The effects of the hormone were rapid, occurring within 9 min, and they did not require new gene transcription or protein synthesis. Moreover, we found that the ER agonist DES and the ER antagonist ICI 182780 had no effect. This is also the case for $E_2$-dependent activation of PKC [60]. In contrast to the inability of DES or ICI 182780 to alter the response to $E_2$, tamoxifen blocked the activation of MAPK. Tamoxifen may act both as an agonist or antagonist of $E_2$ action depending on the response being assayed [13,30]. It is unlikely that it acted via a classical ER pathway in the growth zone cells, however. Tamoxifen is a known PKC inhibitor [46,60] and it blocks the stimulatory effects of $1\alpha,25(OH)_2D_3$ on PKC in both male and female growth zone chondrocytes [60]. Thus, it is probable that it inhibited the effects of $E_2$ through its inhibition of PKC. These observations indicate that sex-specific increases in MAPK occur via non-traditional pathways, and suggest the possibility of additional receptors for the hormone. Novel receptors for $E_2$ that mediate rapid actions of the hormone have recently been identified [20,36], including a receptor expressed only in female cells [17].

Our results show that increased MAPK activity may result from more than one form of the enzyme. At least part of the sex-specific increases in MAPK measured in this study was due to rapid activation of ERK1/ERK2. Western blots indicated that total ERK1/ERK2 was unaffected, but $E_2$ treatment caused a rapid dose-dependent phosphorylation of ERK1/2. Northern analysis supported the lack of a genomic response as there was no change in ERK1 or ERK2 mRNA. Our results are in agreement with those of Doolen et al. [16], demonstrating that PKC and ERK1/2 MAPK are activated in a sex-specific manner in rat intestinal cells. Other investigators have reported that in human osteoblasts, ERK1/2 MAPK is activated by $E_2$ via non sex-specific mechanisms involving ERα [28,29]. Failure to find sexually dimorphic responses may be due to differences in experimental design, differences in cell sources, or differences in species. It should be noted, however, that female-specific increases in $E_2$-dependent PKC have also been reported for human articular chondrocytes [27].

Our results show that effects of $E_2$ on post-natal growth plate chondrocytes are mediated by at least two forms of MAPK. Both PD98059 and SB203580 blocked the $E_2$-dependent stimulation of alkaline phosphatase activity, a hallmark of differentiated growth zone cells. Previous studies have shown the importance of ERK1/2 and p38 for the differentiation of prechondrocytes [52] and the maturation of micromass chondrocyte cultures [16]. This suggests that the two forms of the enzyme work in concert and that $E_2$ induces endochondral maturation in the female growth plate through these interactive pathways. Cross talk between ERK1/2 and p38 pathways has been reported in chondrocytes in response to a variety of regulatory factors [43,66]. Our results support this by demonstrating that the two MAPK families regulate comparable phenotypic responses in the cells.

PD98059 is a widely used inhibitor of ERK family MAPKs. It acts upstream of ERK, preventing its phosphorylation by inhibiting MEK. We did not attempt to assess which isoform of ERK is responsible for the $E_2$-response. Similarly, there are four mammalian p38 genes, all of which are expressed during endochondral development [65]. Because we used a general inhibitor of p38 [75], we were not able to determine which of the p38 isoforms was responsible. It may be that the cooperative effects of ERK1/2 and p38 are due to specific species of each MAPK family as noted for other kinase families.

PD98059, and to a lesser extent SB203580, also reduced the stimulatory effect of $E_2$ on proteoglycan production, based on the inhibition in [$^{35}$S]-sulfate incorporation. Sulfation of glycosaminoglycans in proteoglycan aggregate is a terminal step in their production, occurring after aggrecan and link protein synthesis and glycosylation, a processing requiring numerous enzymes, which themselves must be synthesized and/or activated. Thus, our observation suggests that one or more of these $E_2$-regulated steps are mediated by mechanisms involving transcriptional control via pathways that may be independent of traditional ERs.

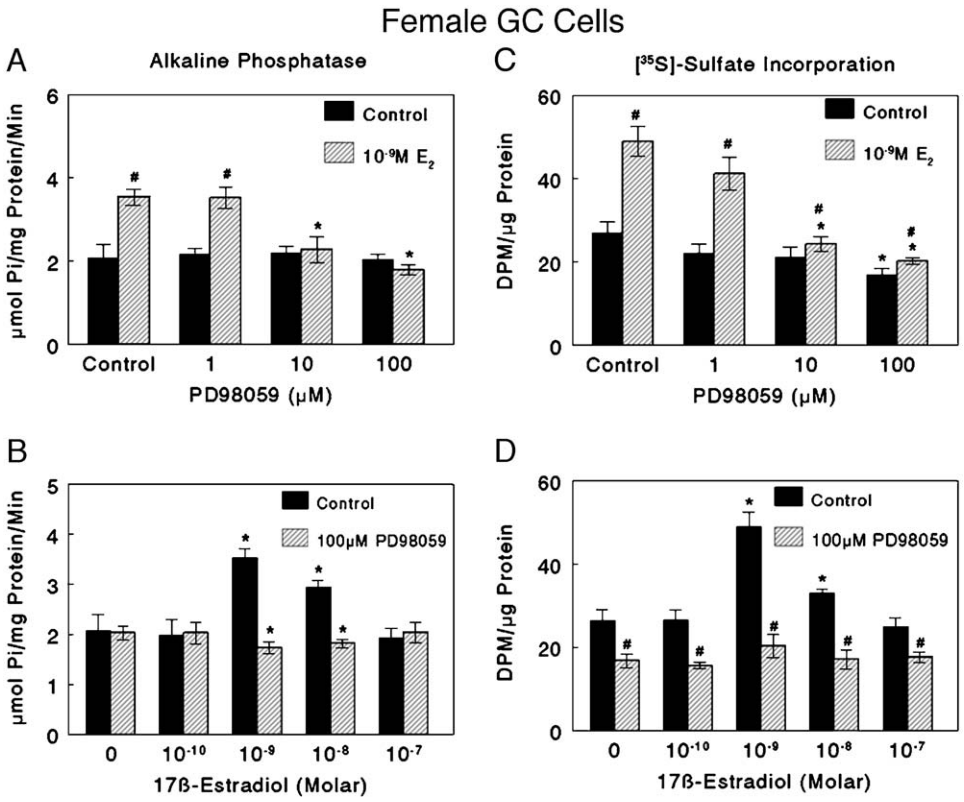


Fig. 7. Effect of PD98059 on alkaline phosphatase-specific activity and [$^{35}$S]-sulfate incorporation in confluent cultures of female rat growth zone (GC) chondrocytes. Cells were treated with 1, 10 or 100 µM PD98059 in the presence of $10^{-9}$ M 17β-estradiol (A, C). Alternatively, cells were treated for 24 h with $10^{-9}$ to $10^{-7}$ M 17β-estradiol in the presence of 100 µM PD98059 (B, D). Values are means ± S.E.M. for six independent cultures. Data are from one experiment of two experiments, both with comparable results. *$P<0.05$ vs. control; $^{\#}P<0.05$ vs. treatment with $10^{-9}$ M 17β-estradiol or 100 µM PD98059.

Our results using post-natal rat growth plate chondrocytes support previous reports using prechondrocyte murine cell models. ERK1/2 and p38 are required for proteoglycan production in cultures of ATDC5 cells [76]. Others [3,79] have shown that inhibition of p38 signaling blocks expression of type X collagen, suggesting that this MAPK is required for chondrocyte hypertrophy. Moreover, p38 inhibition is needed for endochondral development of primary mouse mesenchymal cells [65,66]. In the post-natal rat growth plate, hypertrophy is associated with a reduction in proteoglycan production. Thus, our results suggest that ERK1/2 and p38 control post-proliferative prehypertrophic cells in the zone of maturation when matrix production is intact and alkaline phosphatase enriched matrix vesicles are being released into the extracellular matrix.

JNK MAPK has been shown to be regulated by $E_2$ in other systems [29]. However, inhibition of JNK had no effect on alkaline phosphatase activity or [$^{35}$S]-sulfate incorporation in the growth plate chondrocyte cultures. This suggests that factors controlling these two parameters are not mediated by the JNK signaling pathway.

We showed previously that inhibition of PKC blocks DNA synthesis in growth plate cells, as do treatment with $E_2$ or $E_2$-BSA [68]. Both estrogen compounds activate PKC in resting zone and growth zone cells. In contrast, $E_2$ stimulates proliferation of breast cancer cells [8] and articular chondrocytes [27] via mechanisms involving traditional receptors. This may explain why inhibition of PKC-dependent MAPK activity with PD89059, SB203580, or SP600125 did not have a clearly demonstrable effect on DNA synthesis in the growth plate chondrocytes in response to $E_2$. MAPK pathways are involved in regulation of DNA synthesis, however, as all three inhibitors decreased [$^3$H]-thymidine incorporation in control cultures to some degree, and one or more of the MAPKs may have contributed to the inhibition caused by $E_2$.

There appears to be a relationship between the length of time MAPK remains active and the overall phenotypic response of chondrocytes to factors that regulate MAPK activation. In studies using the RCS chondrocyte cell line to study FGF signaling, rapid transient increases in MAPK were associated with proliferation, whereas persistent elevation in MAPK was associated with chondrocyte growth arrest and differentiation [52]. We found that MAPK activity in general, and ERK1/2 activation specifically, was increased within 9 min. While effects of $E_2$ on ERK were rapid, it is possible that p38 and JNK were activated at a later time. Peak MAPK activity was at 9 min, but activity remained elevated for 180 min. This is long enough for the signaling pathways to modulate existing enzymes within the cells and to initiate new gene expression leading to matrix synthesis and endochondral maturation.

In summary, our findings support our previous findings and the observations of others that membrane-dependent signaling is initiated by $E_2$ via activation of PKC and PKC-dependent MAPK. One of the MAPKs contributing to the rapid increase in

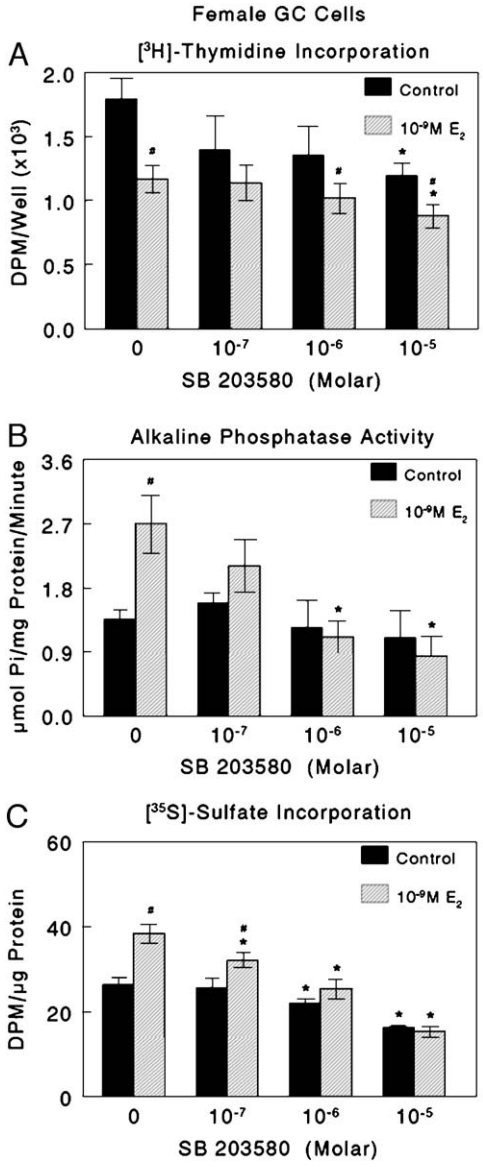


Fig. 8. Effect of SB203580 on [$^3$H]-thymidine incorporation (A), alkaline phosphatase-specific activity (B) and [$^{35}$S]-sulfate incorporation (C) in cultures of female rat growth zone chondrocytes. Cells were treated for 24 h with $10^{-7}$ to $10^{-5}$ M SB203580 in the presence of $10^{-9}$ M 17β-estradiol. Values are means±S.E.M. for six independent cultures. Data are from one experiment of two experiments, both with comparable results. *$P<0.05$ vs. control; $^{\#}P<0.05$ vs. 17β-estradiol treatment.

activity is ERK1/2 and inhibition of this form of MAPK blocks the stimulatory effects of $E_2$ on alkaline phosphatase and [$^{35}$S]-sulfate incorporation. Inhibition of p38 also reduces the stimulatory effects of $E_2$ on these parameters, suggesting that there is cross-talk between these two signaling pathways.

The ERK1/2 MAPK pathway studied here is operative in female cells but not in male cells and does not appear to be mediated by traditional ER mechanisms. Male rat costochondral growth plate chondrocytes possess cytosolic estrogen receptors based on specific binding of radiolabeled $E_2$ but there are fewer cytosolic receptors than in female cells [41]. Assuming that only a subset of these receptors participates in direct PKC activation [35], failure to observe an effect of $E_2$ in male cells may simply reflect a subthreshold response. It is more likely, however, that one or more components of the membrane-initiated signaling pathway is absent in male cells, as we have noted for membrane-mediated activation of PKC by $1\alpha,25(OH)_2D_3$ in resting zone cells [56]. Alternatively, the membrane receptor(s) initiating the signaling cascade may be is present in reduced amounts or absent completely.

Longitudinal bone growth is regulated by estrogen via traditional estrogen receptor pathways [21] in addition to the MAPK signaling cascades described in this paper. At least part of the estrogen effect is sex-specific [50,59]. Our results suggest that MAPK signaling is involved, but other factors may play a

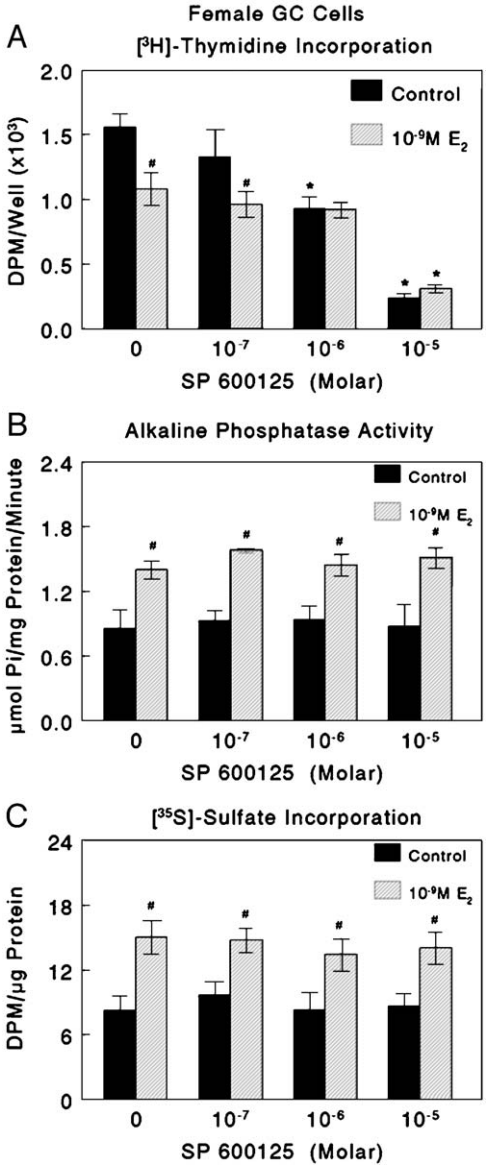


Fig. 9. Effect of SP600125 on [$^3$H]-thymidine incorporation (A), alkaline phosphatase-specific activity (B) and [$^{35}$S]-sulfate incorporation (C) in cultures of female rat growth zone chondrocytes. Cells were treated for 24 h with $10^{-7}$ to $10^{-5}$ M SP600125 in the presence of $10^{-9}$ M 17β-estradiol. Values are means±S.E.M. for six independent cultures. Data are from one experiment of two experiments, both with comparable results. *$P<0.05$ vs. control; $^{\#}P<0.05$ vs. 17β-estradiol treatment.

role. Aromatase-deficient males lack the growth arrest typical of normal males, due to their inability to convert testosterone to estrogen. This may also be a contributing factor to the results presented here. Female chondrocytes express higher levels of aromatase than male cells and produce greater amounts of 17β-estradiol [69]. In contrast, male chondrocytes convert testosterone to dihydrotestosterone via 5-alpha reductase type I [69]. Thus, local concentrations of the sex steroids, as well as systemic levels of the hormones may contribute to the sex-specific regulation of these cells.

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